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Real-Time PCR as a Tool to Study Weed Biology

Wun S. Chao*

Real-time polymerase chain reaction (real-time PCR), also known as quantitative PCR, is used to determine relative gene expression or to quantify exact levels of mRNA in cells or tissues. Before the advent of real-time PCR, the major difficulty associated with traditional quantitative or semiquantitative PCR was to ensure that PCR reactions were quantified within the exponential phase of amplification. Real-time PCR alleviates that problem by detecting and quantifying fluorescent signals after each amplification cycle. Additionally, it does not require running gels and thus is able to produce data in 2 to 3 h. Four different types of chemistries, DNA-binding agents (SYBR Green), hydrolysis probes (TaqMan), hairpin probes (molecular beacons, scorpions), and fluorescent-labeled hybridization probes (Light Cycler), have been commonly used for real-time PCR. Among those chemistries, SYBR Green is the most economical choice. We have used real-time PCR and SYBR Green to examine the expression of a number of leafy spurge genes after growth induction and during normal seasonal growth. Because no reliable endogenous reference genes have been identified in leafy spurge, we performed PCR without an endogenous reference gene and analyzed messenger RNA (mRNA) expression based on the threshold cycle (C_T) value of amplification. Excluding an endogenous reference gene from that data analysis was rather straightforward and reliable if RNA was properly prepared and quantified. Given that genomic tools, such as expressed sequence tags (ESTs), and their expression profiles are lacking for most weedy species, avoiding the use of endogenous reference genes in real-time PCR simplifies the optimization process and reduces the cost tremendously. However, we found that using a passive reference dye (ROX) to normalize non-PCR–related fluctuations in fluorescent signal is desirable.
Nomenclature: Leafy spurge, *Euphorbia esula* L. EPHES.
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Genes that code for proteins are transcribed into messenger RNAs (mRNAs) in the cell nucleus, processed, and transported into the cytoplasm, where the mRNAs are translated into proteins. The expression level of a gene indicates the amount of its corresponding mRNA present in the cell. Many genes change their expression levels in response to various environmental and developmental signals. That process alters cell behavior and is critical for living organisms to survive and thrive in their environment.

Several techniques are used to measure levels of gene expression, including Northern blot analysis (Sambrook et al. 1989), ribonuclease (RNase) protection assay (Sambrook et al. 1989), real-time polymerase chain reaction (real-time PCR) (Higuchi et al. 1993), serial analysis of gene expression (Veculescu et al. 1995), and microarray analysis (Foder et al. 1991; Schena et al. 1995). Conventional techniques to measure single genes, like Northern blot analysis and RNase protection assays, are reliable but are generally labor intensive and require a relatively large amount of RNA to perform the experiment. Serial analysis of gene expression and microarray analysis allow for semiquantitative and simultaneous analysis of a great number of genes; however, specialized and expensive tools are required.

Real-time PCR technology is an improvement of the original polymerase chain reaction (PCR) method developed by Kary Mullis and coworkers (Saiki et al. 1985). Before the advent of real-time PCR, conventional PCR was heavily used to study gene expression. This conventional method is termed quantitative or semiquantitative reverse-transcription polymerase chain reaction (RT-PCR). In RT-PCR, complementary DNAs (cDNAs) are made from mRNAs using deoxynucleotide triphosphates and a reverse transcriptase through the process of reverse transcription (RT). Then, a specific DNA molecule is amplified using PCR via DNA polymerase. The PCR process usually involves 20 to 35 thermal cycles. Each cycle comprises three steps: (1) denaturing at 94 to 96 C to separate hydrogen bonds that connect the two template DNA strands, (2) annealing at 45 to 65 C to attach specific primer pair to the single DNA strands, and (3) extension at 68 to 72 C for DNA polymerase to replicate the specific DNA strands. The major difficulty associated with quantitative or semiquantitative RT-PCR is to ensure that the amplified PCR product is measured within the exponential range of amplification.

Real-time PCR combines amplification and detection into a single step. It does so by detecting and quantifying the fluorescent signal after each amplification cycle. It also produces accurate data with a dynamic range of at least 10^5-fold (Heid et al. 1996) compared with 10^3-fold in conventional RT-PCR. Moreover, it does not require gel electrophoresis and thus is able to produce data in 2 to 3 h. Real-time PCR has been used for pathogen detection, gene expression analysis, single nucleotide polymorphism (SNP) analysis, analysis of chromosome aberrations, quantification of splice variants, and protein detection by real-time immuno-PCR (Adler et al. 2003; Kubista et al. 2006; Valasek and Repa 2005; Vandenbroucke et al. 2001). This article only discusses the application of real-time PCR in gene expression analysis and how this technology can be applied in weed science research.

Fluorescent Chemistries

All real-time PCR systems depend on detecting and quantifying the fluorescent signal, which increases in direct proportion to the accumulation of newly amplified DNA. Four fluorescence-based chemistries are commonly used for quantitative detection (Bustin 2000, Wong and Medrano 2005) (Figure 1). They are DNA-binding agents (SYBR Green), hydrolysis probes (TaqMan), hairpin probes (molecular beacons, scorpions), and fluorescent-labeled hybridization probes (Light Cycler). All chemistries except DNA-binding

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* U.S. Department of Agriculture–Agricultural Research Service, Plant Science Research, 1605 Albrecht Boulevard, Fargo, ND 58105-5674. Corresponding author's E-mail: wun.chao@ars.usda.gov

agents can be used to measure multiple DNA targets in the same reaction (i.e., multiplex PCR) because fluorescent dyes with different emission spectra can be attached to those probe chemistries.

DNA-Binding Agents. The DNA binding agent, SYBR Green, binds only double-stranded DNA and emits fluorescent light upon excitation (Morrison et al. 1998). As shown in Figure 1A, at the beginning of amplification, the unbound dye produces a weak background signal. During annealing and extension of the PCR, more and more dye molecules bind to the newly synthesized DNA, thus an increase in fluorescence can be viewed in real-time. The dye molecules are released and the fluorescent signal falls during denaturing step of the next heating cycle. SYBR Green is inexpensive, easy to use, and sensitive; however, because it binds to any double-stranded DNA molecule in the reaction, including primer dimers (two linked primers) and other nonspecific reaction products, optimization of reactions by dissociation-curve analysis is required (see the section ''Validation of Primers'' for more details).

Hydrolysis Probes. The hydrolysis probes (Figure 1B), such as TaqMan, rely on fluorescence resonance energy transfer (FRET, excitation-caused energy transfer from one dye molecule to the other) for quantification. TaqMan probes are oligonucleotides that anneal to the target sequence between the traditional forward and reverse primers. These probes contain a reporter dye at the 5′-end and a quencher dye at the 3′-end. The quencher dye absorbs the fluorescence of the reporter dye as long as the probe is intact. When the polymerase replicates a template DNA on which a TaqMan probe is bound, the 5′ exonuclease activity of the polymerase cleaves the probe (Heid et al. 1996). This reaction separates the reporter and quencher dye. Once separated by distance, the transfer of energy from the reporter to the quencher can no longer take place, allowing the reporter dye to fluoresce upon excitation by some light source.

Hairpin Probes. The hairpin probes (Figure 1C), such as molecular beacons, also contain reporter and quenching dyes, but FRET only occurs when the quencher dye is adjacent to the reporter dye. Molecular beacons are designed to form a hairpin (stem and loop) structure in solution; the loop of the molecule is complementary to the target DNA, and the stem is formed by annealing of the complementary sequence at the ends of the probe (Tyagi and Kramer 1996). This hairpin structure brings the reporter and quencher dye in proximity, resulting in quenching of the reporter dye. During the denaturation phase, the molecular beacons form a random coil configuration. Upon hybridizing to a target during the annealing and early extension phase of the PCR reaction, the reporter dye and quencher are separated, and the reporter dye emits fluorescence upon excitation. Molecular beacons remain intact during the amplification reaction and rebind to target DNA in every cycle.

Fluorescent-Labeled Hybridization Probes. Fluorescent-labeled hybridization probes (Figure 1D) include two different oligonucleotides (Wittwer et al. 1997). The upstream oligonucleotide is labeled with a donor dye on the 3′-end, and the downstream oligonucleotide is labeled with an acceptor on the 5′-end. These two probes are designed to hybridize adjacently on the single-stranded DNA. When the two dyes are in proximity (one to five nucleotides apart) during the annealing and early extension phase of the PCR reaction, the donor dye will excite the acceptor dye (FRET), resulting in the emission of fluorescence upon excitation. This FRET-mediated excitation system is different from the above-mentioned quenching system, which requires separation of the two dyes for releasing fluorescence.

Data Analysis

The instrumentation platform for real-time PCR consists of a thermal cycler, a computer, optics for fluorescence excitation and emission collection, and data acquisition and analysis software. The basic principle in real-time PCR is that the higher the starting copy number of the target DNA (i.e., reverse-transcribed specific mRNA), the fewer the cycles required to detect a specific threshold level of reporter dye emission. The parameter threshold cycle (C_T) is often used to calculate the levels of target DNA in the sample and is defined as the fractional cycle number at which fluorescence rises above background baseline signal (Figure 2). C_T is always determined during the exponential phase of the reaction when amplification is most efficient. Two approaches are commonly used to quantify gene expression: absolute and relative quantification (Kubista et al. 2006; Livak and Schmittgen 2001; Wong and Medrano 2005).

Absolute Quantification. Absolute quantification determines the copy number of template DNA by relating the fluorescent signal to an absolute standard curve. A standard is a known amount of a target sequence. A set of standards is needed for each target sequence. Single, pure plasmid DNA or in vitro transcribed RNA species with known copy number (converted from A_{260}) is used to generate an absolute standard curve. This curve is then used to quantify the amount of target DNA of unknown samples by interpolation. Absolute quantification should be done where the exact mRNA copy number is required.

Relative Quantification. Relative quantification is adequate for most biological experiments because there is no need to obtain exact copy number in these experiments. Relative quantification determines changes in target gene expression in test samples (i.e., samples of interest) relative to a control (or calibrator) sample, such as an untreated sample or a sample at time zero in a time-course study. Two calculation methods are used for relative quantification including standard curve and comparative C_T.

In the standard curve method, a standard curve is constructed from diluted RNA or DNA. Any stock RNA or DNA containing the relevant target gene can be used to prepare standards. The quantity of target DNA in test samples is first determined from the standard curve and then divided by the quantity of the same target DNA in the control sample. However, if an endogenous housekeeping gene is used for normalization, a separate standard curve is constructed using this endogenous housekeeping gene (see Ambion 2007, for an example).

In the comparative C_T method, C_T values of both the test and control samples are normalized (see the ''Normalization'' section for definition) to an endogenous housekeeping gene.

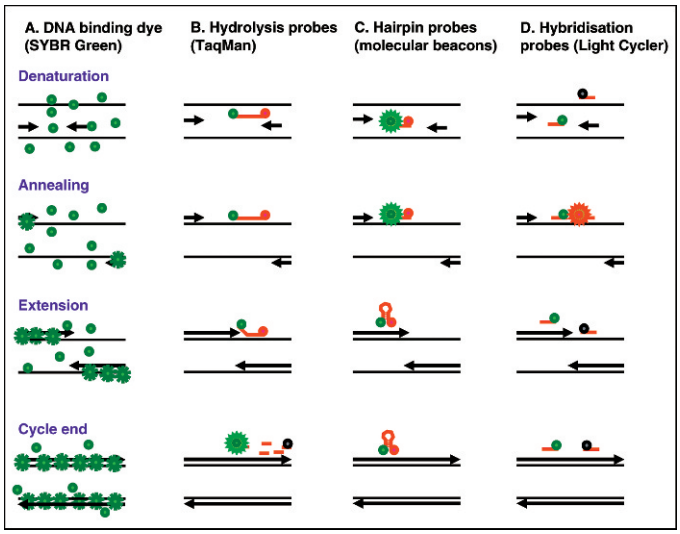

Figure 1. Real-time polymerase chain reaction (real-time PCR) fluorescent chemistries. Figure represents one cycle of amplification. (A–D) Four commonly used fluorescence-based chemistries are shown. DNA binding dyes and oligonucleotide probes are shown in color. Nonfluorescing-dye molecules are indicated by small green, red, or gray circles. Green- or red-fluorescing-dye molecules are indicated by a starburst effect. Primers are indicated by horizontal arrowheads.

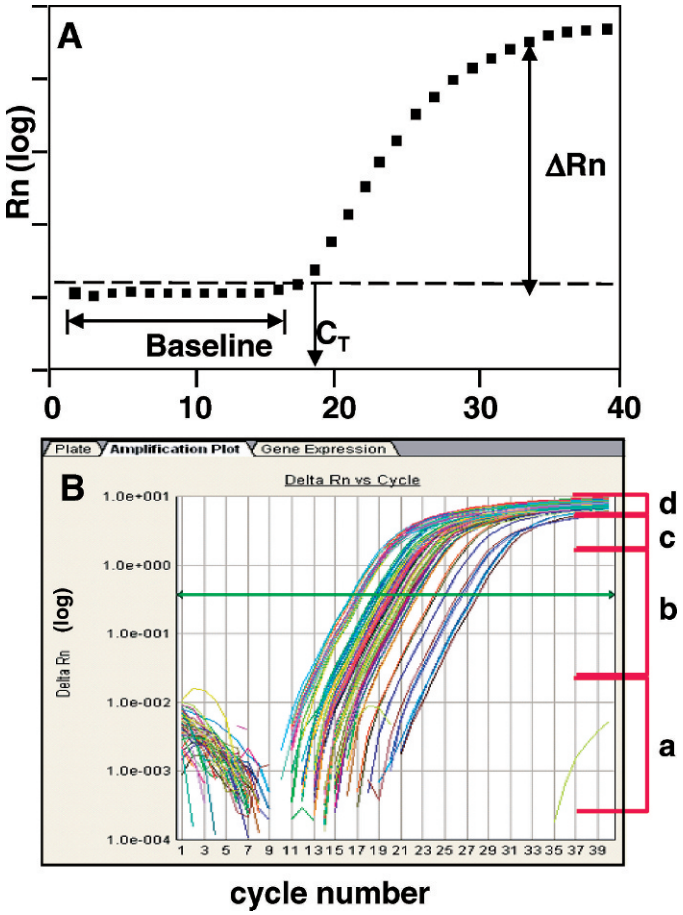

Figure 2. Real-time polymerase chain reaction (real-time PCR) quantifying the fluorescent signal after each amplification cycle. (A) Amplification plot showing the log change in fluorescence (normalized reporter [Rn], log) vs. cycle number. Threshold cycle (C_T) is defined as the fractional cycle number at which the fluorescence rises above the threshold setting. Baseline is the initial cycles of PCR when there is little change in fluorescent signal. Rn is the intensity of the reporter dye fluorescence divided by the intensity of the passive reference dye fluorescence. Passive reference dye such as 6-carboxyl-X-rhodamine (ROX) is used to normalize for non-PCR–related fluctuations in fluorescent signal. Delta Rn (ΔRn) is the magnitude of the signal generated during PCR, and is used to determine C_T (ΔRn = Rn − baseline threshold). (B) The log of ΔRn is plotted vs. cycle number. This graph shows the expression levels of the leafy spurge *histone H3* (*HisH3*) gene based on the results of 96 reactions using 32 complementary DNA (cDNA) samples in three replicates. The threshold (green line) is set in the exponential phase of the amplification curve. Four different phases of amplification are also shown during 40 cycles of PCR—a: background, b: exponential phase, c: linear phase, d: plateau phase.

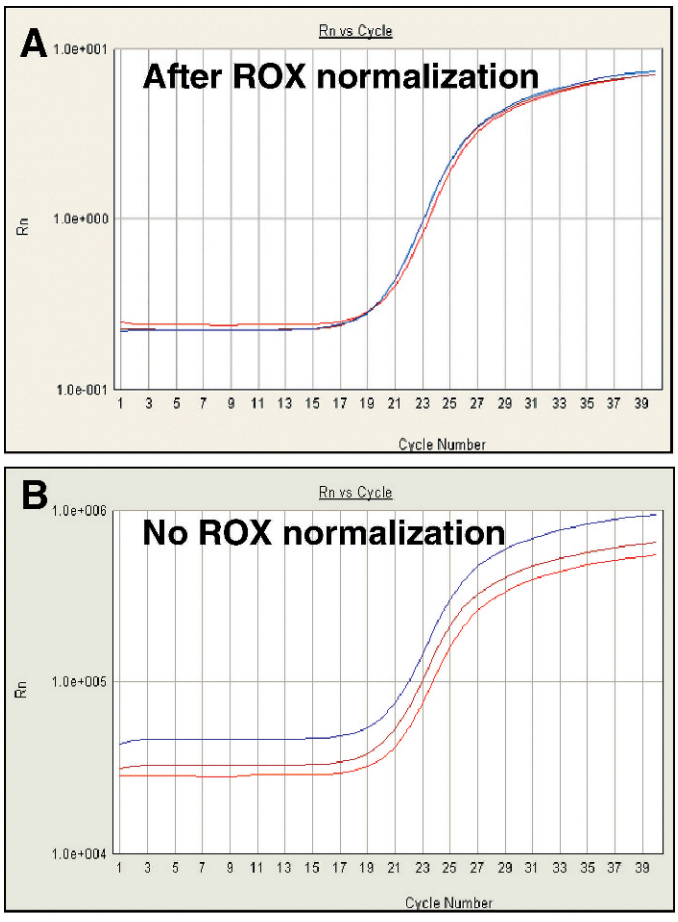

Figure 3. Normalization using passive reference dye (ROX). Amplification plots of a leafy spurge *histone H3* (*HisH3*) gene (in three replicates) are shown (A) with ROX, or (B) without ROX normalization.

The endogenous housekeeping gene is a constitutively-expressed gene that is present in the sample to be analyzed. The normalized C_T values of test samples are then compared with the normalized C_T value of a control sample. The comparative C_T method is also known as the $2^{-\Delta\Delta C_T}$ method, where $\Delta\Delta C_T = \Delta C_{T,test} - \Delta C_{T,control}$. Here, $\Delta C_{T,test}$ is the C_T value of test sample normalized to the endogenous housekeeping gene, and $\Delta C_{T,control}$ is the C_T value of the control normalized to the same endogenous housekeeping gene. The resulting difference in $\Delta\Delta C_T$ is the exponent of base 2 because the amplicon (amplified PCR product) doubles after each amplification cycle. Thus, the $2^{-\Delta\Delta C_T}$ value represents the fold difference in gene expression between control and test samples.

When using the $\Delta\Delta C_T$ method, it is important to determine that amplification efficiencies of the target and the endogenous reference gene must be similar. PCR amplification efficiency is

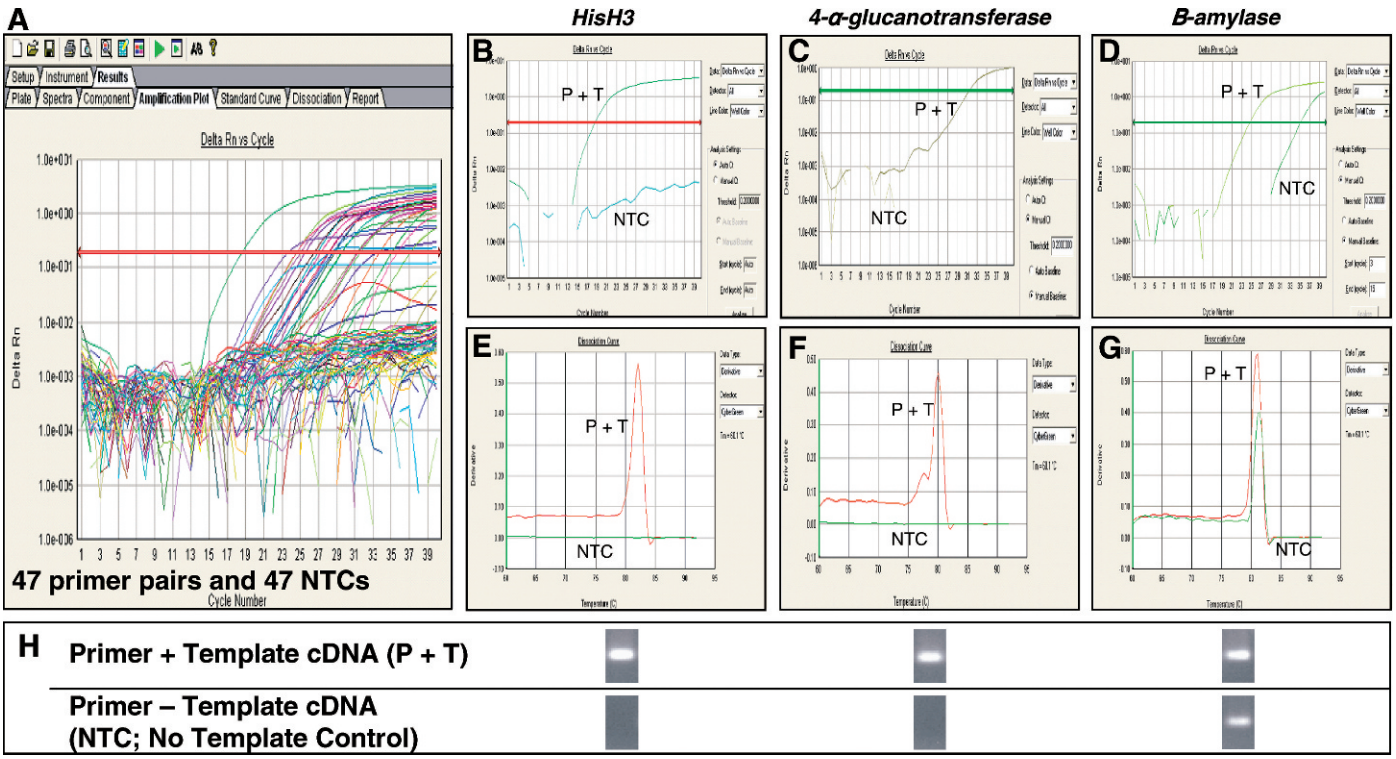

Figure 4. Validation of primers based on amplification plot, dissociation curve, and agarose gel. (A, B, C, and D) Amplification plots are the plots of fluorescent signals vs. cycle numbers. They determine whether the primer pairs can amplify the PCR products. (E, F, and G) The data for dissociation curves are obtained by slowly ramping the temperature of reaction solutions from 55 to 94 C while continuously collecting fluorescent data. Dissociation results are used to determine the specificity of the primers for the target sequence. (H) The end-point PCR product is observed in an agarose gel.

defined as copies (exponential amplification) or percentage (efficiency) of PCR product increase per cycle. It is obtained by performing a 5- or 10-fold serial dilution of the template and plotting log concentration of the template vs. C_T (Ginzinger 2002). The slope of the trend line is a function of the PCR amplification efficiency. The formula for exponential amplification (copies per cycle per template DNA) is $10^{(-1/slope)}$, and the formula for efficiency is $10^{(-1/slope)} - 1$. When PCR is 100% efficient, each cycle produces 2 copies per template DNA, and the *slope* is −3.32 (Ginzinger 2002). A number of factors affect PCR efficiency, including length of the amplicon, secondary structure, and primer design, among others. If a housekeeping gene with a similar amplification efficiency to that of the target cannot be found, the standard curve method can be used instead.

Normalization

Normalization is used to compensate for sample-to-sample variation. Four different strategies have been commonly used for normalizing real-time PCR data (Bustin 2000; Bustin et al. 2005; Huggett et al. 2005; Livak and Schmittgen 2001; Wong and Medrano 2005). Biological normalization uses identical sample amounts to extract RNA or uses an equal quantity of total RNA for reverse-transcription and real-time PCR reaction.

Genetic normalization uses endogenous reference genes. The reference genes can be a housekeeping gene, such as *glyceraldehyde-3-phosphate dehydrogenase* and *β-actin* or a ribosomal RNA, such as 28 S and 18 S. An endogenous reference gene is used to normalize an mRNA target for differences in the amount of total RNA added to each reaction. Because there is no universal reference gene which expressed at a constant level under all conditions and in all tissue types, multiple housekeeping genes are sometimes used to identify the gene most suitable for use as a reference. Microarray analysis can also be exploited to identify potential reference genes for normalization. Czechowski et al. (2005) have identified 18 new genes that are superior for mRNA normalization in *Arabidopsis* gene expression studies based on Affymetrix ATH1 whole-genome GeneChip studies.

Exogenous normalization uses a characterized RNA or DNA as a control and adds it into each sample at a known concentration. This control is used to normalize for differences in the efficiency of sample extraction, cDNA synthesis, and PCR reaction. Finally, passive reference dye, such as 6-carboxyl-X-rhodamine (ROX), is used to normalize for non-PCR–related fluctuations in fluorescent signal including fluorescent fluctuations, well-to-well volume variations, and minor volume differences and changes in concentration (Applied Biosystems 2000). Figure 3 is the amplification plots of *histone H3* (*HisH3*) obtained from one cDNA sample in three replicates. The graphs show that after ROX normalization, the precision of three amplification curves increases dramatically.

The Expression of Leafy Spurge Genes

We have applied this technology to examine the expression of a number of leafy spurge genes. In underground adventitious buds of leafy spurge, carbohydrate contents shift dramatically in response to seasonal signals and growth

induction (after decapitation). For example, during the seasonal cycle, sucrose levels increase in November and December, whereas starch levels decrease progressively from August to December (Anderson et al. 2005). After growth induction, sucrose levels decrease significantly 1 d after decapitation and stay at similar levels until day 5. Starch levels also decrease quickly and continuously from day 1 to day 5 after decapitation (Chao et al. 2006). Because carbohydrates (sucrose and glucose) inhibit the growth of these underground adventitious buds (Chao et al. 2006), it is hypothesized that these sugar molecules have regulatory roles in bud dormancy and growth. Real-time PCR was used to identify differentially regulated genes involved in carbohydrate metabolism and to study expression levels of those genes.

The objective of the following sections is to provide general guidelines for applying real-time technology in weed science research. A cell-cycle gene, *HisH3*, is used as an example on analysis of real-time PCR data. Because the expression patterns of *HisH3* are known in underground adventitious buds of leafy spurge (Anderson et al. 2005; Jia et al. 2006), that gene is also used to check whether total RNA or cDNA samples were prepared adequately.

Experimental Design. Forty-seven primer pairs were designed from 12 carbohydrate metabolism genes and *HisH3* based on the sequence of leafy spurge in expressed sequence tags (EST) database (Anderson et al. 2007). Expression of those genes was examined using total RNA prepared from underground adventitious buds of seasonal samples and growth-induced samples. Seasonal bud samples were harvested monthly (July to December) from field-grown leafy spurge (Anderson et al. 2005), whereas growth-induced bud samples were harvested from greenhouse-grown plants 0 h, 2 h, 4 h, 8 h, 16 h, 1 d, 2 d, 3 d, 4 d, and 5 d after shoot removal (Jia et al. 2006). A total of 32 individual bud samples, including 2 sets of seasonal (2002 and 2003) and 2 sets of growth-induced (2003 and 2004) samples, were used to examine the expression of those genes.

Total RNAs were extracted from 32 bud samples, quantified, and used to prepare cDNA template through RT reaction. Because a reliable endogenous reference gene has not been discovered from leafy spurge for gene expression studies, the biological normalization approach was used. This is done by using the same amount of total RNA for RT and real-time PCR reaction. To reduce the incidence of unexpected sample variation, RNA extractions were done using the same methods. In addition, RNA extraction, concentration measurement, and RT reactions were done at the same time for the set of samples to be compared. Nanodrop instrumentation (a spectrophotometer that is capable of measuring 1-µl samples with good reproducibility) was used to accurately quantify the concentration of total RNA.

Total cDNA was prepared according to the manufacture's (Invitrogen) instructions, and real-time PCR was performed on a 7300 Real-Time PCR System.[1] Briefly, 5 µg of total RNA was DNase[2]–treated, and then, reverse transcription was performed in 20 µl total volume using a SuperScript First-Strand Synthesis Kit[3] to produce total cDNA from each sample. After cDNA synthesis, each reaction was diluted to 800 µl total volume and stored at −80 C. For real-time PCR reactions, 2 µl total cDNA was added to a 20-µl PCR reaction mixture containing 10 µl of 2× Power SYBR Green PCR Master Mix[4] and 0.5 µl of each primer (20 pmol). Thermal-cycling was performed with a thermal profile step of 2 min at 50 C, an auto increment step of 10 min at 95 C, and followed by 40 cycles of 20 s at 95 C, 10 s at various annealing temperatures (50 to 58 C), and 35 s at 72 C. For dissociation analysis, a temperature ramp step was added to the end of the thermal profile with an initial temperature of 55 C and a final temperature of 95 C. PCR reactions were electrophoresed on 1% agarose gels. Primers (21 to 24 nucleotides) were designed using Lasergene sequence analysis software.[5] SYBR Green chemistry was used to produce fluorescent signal. Three technical replicates were used per sample for real-time PCR experiments.

Validation of Primers. Before performing full scale real-time PCR experiments, primer pairs need to be tested based on amplification plot, dissociation curve, and agarose gel. Forty-seven primer pairs were tested in a PCR reaction with or without template cDNA and designated as primer + template (P + T) and no-template control (NTC) reactions, respectively. Template cDNA for this test was made by combining reverse-transcribed cDNA species.

An amplification plot is used to determine whether the primer pair can amplify a PCR product. Figure 4A depicts the amplification plots of 96 reactions, which show that 60% of the 47 primer pairs amplified a PCR product at an annealing temperature of 55 C. The expression levels obtained from three genes, *HisH3, 4-α-glucanotransferase*, and *β-amylase*, are presented (Figures 4B–D) as examples. An expected amplicon is shown in the P + T reactions (Figures 4B and 4C) for *HisH3* and *4-α-glucanotransferase*, whereas the NTC reactions do not have a PCR product (Figures 4B and 4C). For *β-amylase*, an amplicon is shown in P + T and NTC reactions (Figure 4D); the amplicon in NTC reaction is a nonspecific PCR product. The information obtained from these amplification plots indicates that all three primer pairs amplify the expected gene product, but the *β-amylase* primer pair is not very specific. Because the *β-amylase* primer pair also amplifies a nonspecific PCR product, the signal generated from this nonspecific amplicon can inflate the value of the expression level of *β-amylase*. Designing and using a new primer pair could eliminate this problem.

SYBR Green detects any double-stranded DNA (dsDNA). It is therefore important to run a dissociation analysis to ensure that the fluorescent signal is recorded from the desired amplicon. Every dsDNA has a melting point (Tm) where 50% of the DNA becomes single stranded. Therefore, during the dissociation test, the instrument will detect a sudden decrease in fluorescence when the Tm is reached. The results of dissociation analysis are shown in dissociation curves (Figures 4E–G). *HisH3* has one peak in the P + T reaction (Figure 4E), indicating that this peak is obtained from dissociation of the *HisH3* amplicon. The NTC reaction has no peak. *4-α-Glucanotransferase* has two peaks in the P + T reaction (Figure 4F); the smaller (shoulder) peak could result from primer dimers. That shoulder peak can inflate the quantity of the expression level of the target. Designing a new primer pair, optimizing primer concentration by lowering amounts of one or both primers, or increasing the concentration of template DNA can solve this problem. However, in the author's opinion, it may be possible to exclude the nonspecific fluorescent signal of primer dimers by

Table 1. Relative quantification of *histone H3* (*HisH3*) gene expression using seasonal, underground adventitious bud samples.

	Average C_T	$\Delta C_T = C_{T,test} - C_{T,control}$	$2^{-\Delta C_T}$	Log_2
2002				
July	20.982	0	1	0
August	18.869	−2.113	4.327	2.113
September	19.656	−1.327	2.509	1.327
October	19.447	−1.536	2.899	1.536
November	23.420	2.437	0.185	−2.437
December	26.693	5.710	0.019	−5.710
2003				
July	20.794	0	1	0
August	19.817	−0.977	1.968	0.977
September	19.550	−1.244	2.369	1.244
October	20.621	−0.173	1.127	0.173
November	21.449	0.655	0.635	−0.655
December	25.422	4.628	0.040	−4.628

recording the fluorescent data at a temperature between the nonspecific peak and the amplicon peak if those two peaks are clearly separated and the amplicon peak is to the right of the nonspecific peak. Two peaks are observed for *β-amylase*, and these two peaks are overlapping (Figure 4G). The larger peak represents the *β-amylase* amplicon, whereas the smaller one is a nonspecific PCR product obtained from the NTC reaction.

Finally, all PCR reactions were run in a gel to observe the end-point–accumulated PCR product (Figure 4H). The desired scenario for this test is to detect a single band in P + T reaction as either *HisH3* or *4-α-glucanotransferase*. After these tests, good primer pairs were used for gene expression studies.

Data Analysis. The comparative C_T method was used to determine changes in target gene expression in test samples relative to a control sample. The formula used to calculate the fold differences is similar to the aforementioned comparative C_T method except that no endogenous reference gene is incorporated in the calculation. The modified formula for fold difference in gene expression of test vs control sample is $2^{-\Delta C_T}$, where $\Delta C_T = C_{T,test} - C_{T,control}$. The detailed quantification process is shown in Tables 1 and 2 using *HisH3* as an example.

The control for seasonal samples is the July buds (Table 1), and the control for growth-induced samples is 0-h buds (Table 2). Both controls are selected arbitrarily for comparison purpose. The fold difference is designated as $2^{-\Delta C_T}$ and log_2 values in Tables 1 and 2. *HisH3* exhibits the expected expression pattern in two seasonal cycles (2002 and 2003). The mRNA of *HisH3* was up-regulated in August and began to down-regulate in September or October (see Log_2 column in Table 1). The lowest expression levels for both years were in December. In 2002, the log_2 value for December was −5.71, which is 53-fold less than that of July. *HisH3* mRNA also shows the expected expression pattern after growth induction in two biological replicates (2003 and 2004). *HisH3* mRNA started to increase 1 d after decapitation and reached plateau by day 2 (see Log_2 column in Table 2). Because the expression patterns of *HisH3* obtained from real-time PCR analysis are similar to known expression patterns identified previously (Anderson et al. 2005; Jia et al. 2006), the combined results indicated that cDNA samples were prepared adequately and thus are reliable for studying the expression of other genes.

Table 2. Relative quantification of *histone H3* (*HisH3*) gene expression using growth-induced underground adventitious bud samples.

	Average C_T	$\Delta C_T = C_{T,test} - C_{T,control}$	$2^{-\Delta C_T}$	Log_2
2003				
0 h	19.803	0	1	0
2 h	20.411	0.607	0.656	−0.607
4 h	19.189	−0.614	1.531	0.614
8 h	19.092	−0.711	1.637	0.711
16 h	20.127	0.324	0.799	−0.324
1 d	17.800	−2.004	4.010	2.004
2 d	16.406	−3.397	10.536	3.397
3 d	16.731	−3.072	8.411	3.072
4 d	16.455	−3.348	10.182	3.348
5 d	16.489	−3.314	9.945	3.314
2004				
0 h	19.113	0	1	0
2 h	18.596	−0.517	1.431	0.517
4 h	19.098	−0.0153	1.011	0.015
8 h	18.807	−0.306	1.236	0.306
16 h	19.155	0.0418	0.971	−0.042
1 d	17.912	−1.200	2.298	1.200
2 d	16.258	−2.855	7.236	2.855
3 d	16.280	−2.833	7.128	2.833
4 d	16.124	−2.989	7.939	2.989
5 d	16.519	−2.594	6.039	2.594

Conclusion

Real-time PCR technology can be used to identify candidate genes responsible for weedy characteristics, to compare expression levels of specific genes among ecotypes, and to examine the expression of various genes after herbicide treatment and other environmental and developmental stimulations. Several laboratories have used this technology to perform weed science–related research. For example, real-time PCR was used to study the expression of metallothionein and basic region/leucine zipper motif (bZIP) transcription-factor genes following a biocontrol agent, *Colletotrichum coccodes*, infection in velvetleaf (*Abutilon theophrasti* Medik.) (Dauch and Jabaji-Hare 2006) and to study the expression of auxin-responsive genes *9-cis-epoxycarotenoid dioxygenase* (Kraft et al. 2007) and *CH3* (Kelley et al. 2006) after auxin herbicide treatment in cleavers (*Galium aparine* L.) and soybean [*Glycine max* (L.) Merr], respectively. Real-time PCR was also used to identify and quantify herbicide-resistant biotypes in *Lolium* spp. populations based on detecting DNA mutations in the acetyl-coenzyme A carboxylase (ACCase) enzyme (Kaundun et al. 2006).

Although endogenous reference genes would be advantageous for normalizing differences in the amount of total RNA added to each reaction, identifying a universal reference gene can be very challenging (Bustin et al. 2005). Nevertheless, without an endogenous reference gene, real-time technology still can be applied by carefully measuring and maintaining the concentration of total RNA for reverse-transcription and real-time PCR reactions as described in this article. Paradoxically, because the target and endogenous reference genes need to be amplified separately using SYBR Green chemistry, which would increase the amount of template cDNA and the cost of reagents, managing to run real-time PCR without using an endogenous reference gene could be beneficial in this perspective.

Sources of Materials

[1] 7300 Real-Time PCR System, Applied Biosystems, Foster City, CA.

[2] DNase, Invitrogen Corporation, Carlsbad, CA.

[3] SuperScript First-Strand Synthesis Kit, Invitrogen Corporation, Carlsbad, CA.

[4] Power SYBR Green PCR Master Mix, Applied Biosystems, Foster City, CA.

[5] Lasergene sequence analysis software, DNASTAR, Inc., Madison, WI.

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